# Therapeutic potential of Beta-caryophyllene combined with L-arginine in the management of diabetic complications

SUMMARY FOR THE DEGREE OF

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**Candidate**

**Mr. Vivek S. Kumawat**

**Supervisor**

**Dr. Ginpreet Kaur**

**Shobhaben Pratapbhai Patel**

**School of Pharmacy & Technology Management**

**SVKM's Narsee Monjee Institute of Management Studies**

**V.L. Mehta Road, Vile Parle (W), Mumbai-40005**

**Introduction:**

Diabetes mellitus is a chronic metabolic disorder characterized by persistent hyperglycemia resulting from impaired insulin production, secretion, or utilization. With its prevalence steadily rising worldwide, diabetes mellitus has become a significant global health challenge [1]. In 2021, the global burden of diabetes mellitus remains a pressing concern, with an estimated 537 million individuals affected by the condition. Alarmingly, this number is projected to increase to 643 million by 2030 and a staggering 783 million by 2045 [2]. Moreover, an additional 541 million people are estimated to have impaired glucose tolerance, predisposing them to develop diabetes. The importance of the situation is further highlighted by the prediction that over 6.7 million people between the ages of 20 and 79 will succumb to diabetes-related causes in 2021 alone. The economic implications of diabetes are immense, with direct health expenditures already approaching one trillion USD [3,4]. Alarmingly, this figure is expected to exceed this threshold by 2030, putting additional burden on healthcare systems worldwide. Diabetes mellitus encompasses several distinct types, each characterized by specific causes and mechanisms. Type 1 Diabetes Mellitus (T1DM) is an autoimmune condition where the immune system targets and destroys the pancreatic beta cells responsible for insulin production. This results in a deficiency of insulin and requires lifelong insulin therapy for proper blood sugar management. Type 2 Diabetes Mellitus (T2DM) is the most prevalent form, arising from a combination of insulin resistance (where cells become less responsive to insulin) and inadequate insulin production by the pancreas. Gestational diabetes occurs exclusively during pregnancy, characterized by hormonal changes that lead to insulin resistance and elevated blood glucose levels [5].

Chronic hyperglycemia, characterized by persistently high blood sugar levels, can cause significant harm to vascular organs in the body, leading to complications like diabetic nephropathy,

neuropathy, cardiomyopathy, retinopathy, and cerebral stroke [6]. The increased production of intracellular advanced glycation end products (AGEs) is one of the key mechanisms by which chronic hyperglycemia contributes to these complications. Additionally, uncontrolled hyperglycemia results in the overproduction of reactive oxygen species (ROS), which are highly reactive molecules causing oxidative stress. These processes collectively activate major pathological pathways, including the protein kinase-C (PKC) pathway, hexosamine pathway, and increased polyol flux. The activation of these pathways, along with the ensuing inflammatory cascade and cellular damage, plays a significant role in the development of complications associated with chronic hyperglycemia [7].

Recent advances in the management of diabetic complications have brought attention to the endocannabinoid system (ECS) due to its regulatory role in various physiological processes, including metabolism, inflammation, and neuroprotection [8]. Dysregulation of the ECS has been implicated in the development and progression of diabetic complications. Research has shed light on the role of cannabinoid receptors, specifically the $CB_2$ receptor, which has emerged as a potential therapeutic target [9]. The dysregulation of cannabinoid receptors, particularly $CB_2$, has been associated with diabetic nephropathy, neuropathy, retinopathy, and cardiomyopathy [10]. Activation of $CB_2$ receptors using specific agonists has shown promising effects in reducing kidney damage, alleviating neuropathic pain, reducing retinal damage, and improving cardiac function in animal models of diabetes [11]. Additionally, cannabinoid receptors have been linked to the regulation of inflammation and metabolic processes in diabetes [12]. Modulating cannabinoid receptor signaling holds potential for managing inflammation and modifying metabolic imbalances in diabetic complications. It's worth noting that $CB_2$ receptors are primarily located in peripheral tissues, particularly immune cells, rather than in the central nervous system (CNS) [13,14].

This peripheral distribution makes targeting CB2 receptors potentially safer in terms of psychoactive effects compared to $CB_1$ receptors [15]. Preclinical and some clinical studies support the potential of $CB_2$ receptor modulation in diabetic complications, highlighting its promising role in their management [16].

Nitric oxide (NO) plays a crucial role in diabetic complications. In diabetes, there is a dysregulation of NO production and bioavailability, leading to various adverse effects [17,18]. Reduced NO bioavailability contributes to impaired endothelial function, vasoconstriction, and increased oxidative stress, leading to vascular complications such as nephropathy, retinopathy, and cardiovascular diseases. Endothelial dysfunction, impaired angiogenesis, and nerve damage are also consequences of impaired NO signaling [19,20]. Moreover, dysregulated NO production can contribute to chronic inflammation and tissue damage in diabetes. In diabetic nephropathy, reduced NO availability contributes to renal vasoconstriction and fibrosis, impairing kidney function. Strategies to improve NO bioavailability and function are being explored as potential therapeutic approaches for managing diabetic complications. These include using NO donors, antioxidants, and modulators of NO synthase activity [21].

The management of diabetic complications poses a significant challenge due to the heterogeneous nature of the disease, variations in prevalence across geographical regions, and involvement of multiple pathways. Current approaches to manage complications such as nephropathy, neuropathy, cardiomyopathy, and retinopathy are limited in their effectiveness and often associated with side effects and high costs [22]. Therefore, there is a need to search for alternative approaches that are more economic, efficient, and safe.

One potential avenue for managing diabetic complications is through targeted interventions that address the underlying pathophysiological mechanisms. As mentioned earlier, the dysregulation

of pathways such as the endocannabinoid system, oxidative stress, inflammation, and impaired nitric oxide bioavailability play significant roles in the development and progression of diabetic complications. By targeting these specific pathways, it may be possible to intervene and attenuate the complications associated with diabetes. Additionally, natural products and compounds derived from plants have gained attention for their potential therapeutic effects in managing diabetic complications [23]. These natural products often contain bioactive compounds with antioxidant, anti-inflammatory, and anti-fibrotic properties.

Beta-caryophyllene is a natural bicyclic sesquiterpene compound that belongs to the class of chemicals known as terpenes [24]. It is found in various plants, particularly in essential oils derived from spices such as black pepper, cloves, and cinnamon [25]. One interesting aspect of beta-caryophyllene is its interaction with the ECS While it does not directly bind to cannabinoid receptors like THC or CBD, it acts as a selective agonist for the CB2 receptor [26]. Due to its interaction with the CB2 receptor, beta-caryophyllene has gained attention in the field of cannabinoid research. It is considered a phytocannabinoid, a cannabinoid-like compound found in plants. Researchers have been exploring its therapeutic potential in various areas, including pain management, inflammation reduction, and neuroprotection [27,28]. Moreover, beta-caryophyllene has been studied for its potential benefits in other health conditions. Research suggests that it may have antioxidant [29], antimicrobial [30], and anticancer properties [31]. It has also shown promise in preclinical studies for its potential to protect against gastric ulcers [32] and alleviate anxiety and depression-like behaviors [33].

L-arginine is a semi-essential amino acid that plays a crucial role in various physiological processes in the human body. It is obtained through dietary sources and can also be synthesized by the body [34]. LA is involved in protein synthesis, the production of nitric oxide (NO), and the

urea cycle. LA is also a precursor for the production of nitric oxide, a molecule that acts as a signaling molecule in the body [35]. Nitric oxide helps relax and dilate blood vessels, thereby promoting healthy blood flow and supporting cardiovascular health [36]. In addition to these essential roles, LA has been investigated for its potential health benefits in various conditions. Research suggests that it may have antioxidant properties, support immune function, and play a role in hormone regulation [37].

Individually, both beta-caryophyllene LA have been investigated for their potential health effects. While there is no specific evidence on the combination of beta-caryophyllene and LA for diabetic complications, it is theoretically possible that their individual properties could have complementary effects. BCP's potential anti-inflammatory and antioxidant properties, combined with LA has potential to improve vascular function, could potentially provide benefits in reducing inflammation, oxidative stress, and improving blood circulation. In this study, we investigated the potential effects of combining BCP and LA in the context of STZ induced diabetic complications. Our aim was to evaluate the therapeutic outcomes of this combination therapy and explore the underlying mechanisms, specifically focusing on the involvement of $CB_2$ receptors and nuclear factor- κβ (NF-κβ) pathway in managing diabetic complications. Furthermore, we compared the individual treatments of BCP and LA with the combination therapy to assess their relative efficacy and potential synergistic effects.

**Rationale:**

Diabetes mellitus is a global health challenge with a steadily increasing prevalence. The current approaches to managing diabetic complications have limitations in terms of their effectiveness and potential side effects. Therefore, alternative therapeutic strategies are necessary to address this

pressing health concern. The development and progression of diabetic complications involve a complex interplay of various mechanisms and pathways. Increased inflammation and oxidative stress are hallmark features resulting from persistent hyperglycemia [38]. These processes contribute to tissue damage, vascular dysfunction, and organ dysfunction, which are characteristic of diabetic complications. The dysregulation of the ECS has been implicated in the pathogenesis of diabetic complications. Activation of cannabinoid receptors, particularly the $CB_2$ receptor, has shown promising effects in reducing kidney damage, alleviating neuropathic pain, reducing retinal damage, and improving cardiac function in animal models of diabetes. Modulating the ECS could provide a novel therapeutic approach to managing diabetic complications. In addition, in diabetes, there is a dysregulation of NO production and bioavailability, leading to adverse effects such as impaired endothelial function, vasoconstriction, and increased oxidative stress. Restoring NO bioavailability can improve endothelial function and vascular health, reducing the risk of diabetic complications [12].

BCP, as a natural selective $CB_2$ agonist, possesses antioxidant and anti-inflammatory properties, which can help mitigate oxidative stress and inflammation associated with diabetic complications. LA, as a precursor for NO production, can enhance NO bioavailability, improving endothelial function and vascular health. BCP, as a natural selective CB2 agonist, possesses antioxidant and anti-inflammatory properties, which can help mitigate oxidative stress and inflammation associated with diabetic complications. LA, as a precursor for NO production, can enhance NO bioavailability, improving endothelial function and vascular health.

The study focused on understanding the involvement of $CB_2$ receptors and the NF-κβ pathway. $CB_2$ receptors are primarily located in peripheral tissues, particularly immune cells, and modulating their activity can regulate inflammation and immune responses. The NF-κβ pathway

plays a key role in the pathogenesis of vascular complications of diabetes, and inhibiting its pro-inflammatory effects is an important target for management.

By evaluating the efficacy of the combination therapy of BCP and LA and investigating the involvement of CBβ receptors and the NF-κβ pathway, the study aimed to provide insights into the underlying mechanisms and potential therapeutic benefits of this approach. The results of this study may contribute to the development of novel and more effective strategies for managing diabetic complications, addressing the limitations of current approaches, and improving patient outcomes.

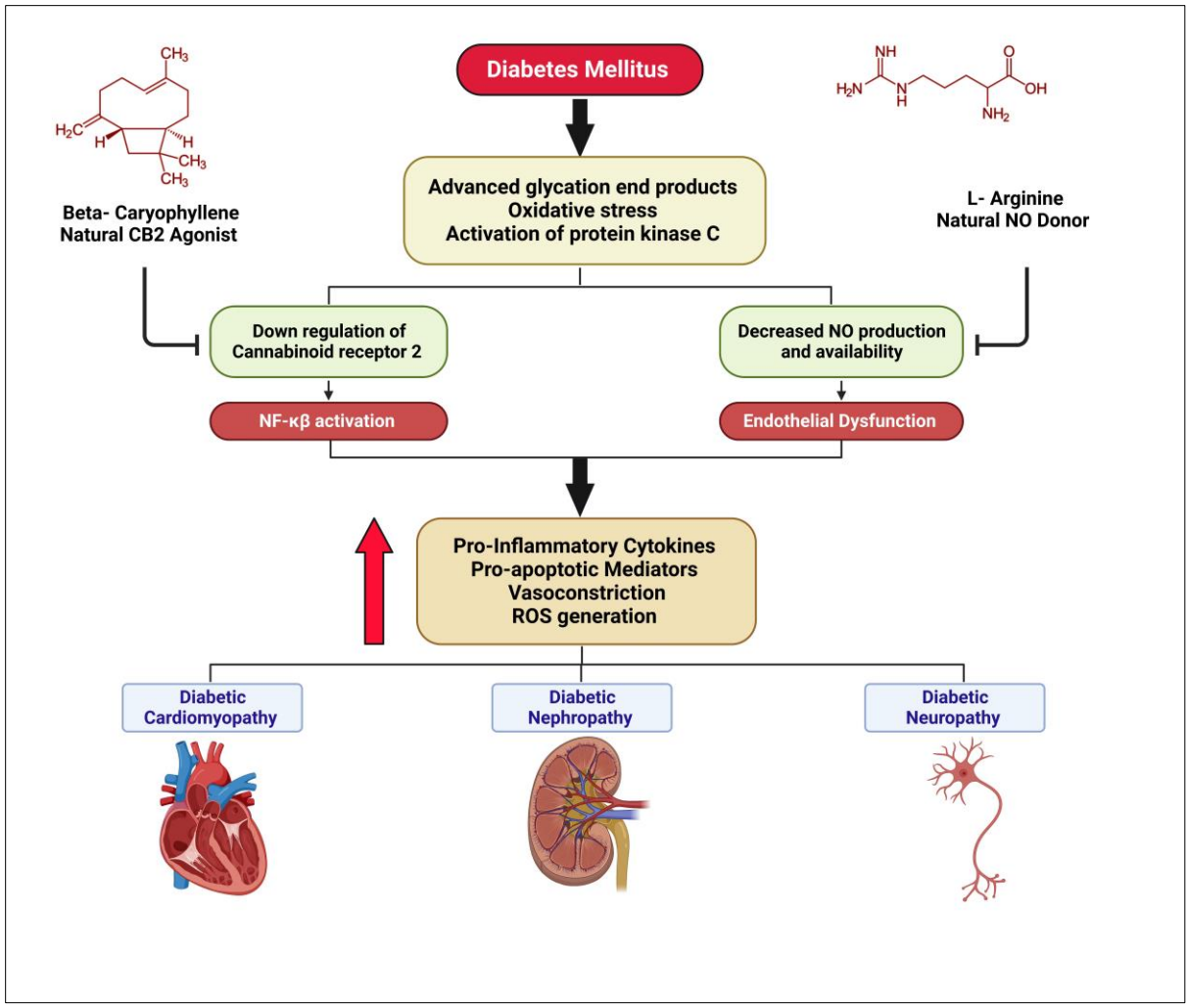


*Figure 1 The treatment approach of diabetic complications through BCP and LA combination*

**Objectives**

1. To study the effects of a CB2 agonist (β-caryophyllene) and L-arginine combination on an inflammatory marker (TNF-α) on cell line (RAW 264.7)

2. To study the effect of a CBβagonist (β-caryophyllene) and L-arginine combination in experimental models by streptozotocin-induced diabetes complications.

i) Diabetic Nephropathy

ii) Diabetic Neuropathy

iii) Diabetic Cardiomyopathy

3. To study the anti-oxidant potential of a CB2 agonist (β-caryophyllene) and L-arginine in heart, kidney, and sciatic nerve.

4. Exploring the anti-inflammatory potential of a CB2 agonist (β-caryophyllene) and L-arginine by inflammatory markers such as Nf-κβ, TNF- α.

5. To study the expression of CB2 receptors in the kidney and heart by immunohistochemistry.

**To study the effects of Combination of Beta-caryophyllene and L-arginine on inflammatory markers on RAW 264.7 cell lines.**

**i. LPS-induced inflammation in RAW 264.7 macrophage cells:**

Murine macrophage RAW 264.7 cells (National Centre for Cell Science (NCCS) Pune (India)) cultured in Dulbecco's Modified Eagle Medium (DMEM) (Himedia Laboratories Limited, India) media supplemented 10% v/v fetal bovine serum (FBS) (Himedia Laboratories Limited, India), penicillin (100 units/mL), and streptomycin (100 mg/mL) (Himedia Laboratories Limited, India), in incubator (5% CO2) at 37°C temperature**.**

**ii. Estimation of inflammatory cytokines (IL-6 and TNF-α) in Lipopolysaccharide- induced inflammation in RAW Macrophage cells:**

RAW macrophage cells were cultured in 96-well plates with 5X103 cells each well, kept an incubator with carbon dioxide (CO2) at 37° for 24 hours to let them to adhere. RAW macrophage cells treated with BCP (Sigma-Aldrich, USA), LA (Arrobiochem India) and a combination of both (100, 200 and 500 μM) for 1 hr. to prior addition of Lipopolysaccharides (LPS) (Sigma-Aldrich, USA) 1 μg/ml in each well except control well for 24 hrs. After 24 hrs. treatment of drugs and

LPS, 100 µl supernatant was kept in freeze (-80°C) till evaluation. TNF-α and IL-6 were estimated by commercial kits (BT-bioscience) and cell supernatant was used as the sample (Gao et al., 2012).

**Experimental Animals:**

The experiments utilized male Sprague Dawley (SD) rats (180-200 grams) from the National Institute of Biosciences in Pune. Experimental animals were kept at the Shri Vile Parle Kelwani Mandals Narsee Munjee Institute of management studies (SVKM's NMIMS) animal house facility with an ambient temperature of 22°C ±1°C, humidity (55 ± 5%), and a 12-hour dark/night cycle. Animals have unrestricted water and a pellet diet. The Institutional Animal Ethics Committee (IAEC), under the CPCSEA, India, accepted the study protocol.

**To study the effect of Combination of Beta-caryophyllene and L-arginine in an experimental model of diabetic nephropathy:**

**Induction of diabetes and treatment:**

A single dosage of STZ (55 mg/kg, i.p. Sigma-Aldrich, St. Louis, MO, USA) was used to induce the DCM. Animals with plasma glucose levels (PGL) more than 250 mg/dl were included in the study. Animals were randomly divided into groups of eight animals each after four weeks. One group of non-diabetic animals was kept as normal control while one group of diabetic animals was kept as diabetic control, other groups received lisinopril (05 mg/kg), BCP (200 mg/ kg), LA (100 mg/kg), COM1- BCP (100 mg/kg) and LA (100 mg/kg) and COM2- BCP (200 mg/kg) and LA (200 mg/kg) after 28 days of treatment following parameters were evaluated.

Parameters Assessed:

Body weight and relative organ weight:

At the conclusion of the research study, the body weight of each animal was determined with the help of an electronic weighing balance. Animals were humanely sacrificed and both kidneys were isolated carefully. Kidneys were de-capsulated, washed with normal saline solution and weighed to determine relative kidney weight. The relative weight of the kidneys was computed with formula: Relative kidney mass = (Kidney mass / Body mass) * 100.

Biochemical parameters:

Plasma samples were analyzed for biochemical parameters. Blood was drawn from retro-orbital plexus and placed in tubes comprising-coagulant (10% w/v disodium salt of ethylenediaminetetraacetic acid (EDTA). Samples were subjected to centrifuge (2500 rpm) at 4℃ for 15 minutes to separate the plasma. Commercially available diagnostic kits (TransAsia Biomedicals Ltd, India) were used to analyses the samples for glucose, creatinine, total proteins, albumin, blood urea nitrogen (BUN).

Urine analysis:

Commercially accessible diagnostic kits were used to analyses the urine samples for volume, urine protein, albumin, creatinine, and urea (Transasia Biomedicals Ltd, India).

Inflammatory Markers:

TNF-α and IL-6 levels in the plasma were assessed using commercially available ELISA kits (BT Biosciences, China) and a multi-mode microplate reader (Epoch 2, BioTek Instruments, Inc., USA) in accordance with methods provided by the respective manufacturers.

Assessment of renal oxidative stress parameters:

Reduced glutathione (GSH) activity estimated as per the method described in (Ellman, 1959) and Malondialdehyde (MDA) activity estimated in accordance the method (Ohkawa et al., 1979).Post-

mitochondrial supernatant of renal tissue was used to superoxide dismutase (SOD) (Paoletti et al., 1986) and catalase (Lück, 1965) activity.

Renal tissue nitrite levels:

The method described by Gandhi et al. was used to estimate the renal nitrite level. Sulfosalicylic acid was used to transform the tissue homogenate into a protein-free supernatant. The 200 µL supernatant was added with 30 µL of 10% NaOH. Next, 530 µL of Griess reagent was added, and it was incubated in the dark for 10 to 15 minutes. Following incubation, absorbance of sample was measured at 540 nm in comparison to a blank for the Griess reagent. The standard curve of sodium nitrite was used to calculate the unknown concentration of nitrite in the kidney tissue (Gandhi et al., 2009) .

Renal NF-κβ and $CB_2$ expression by western blotting:

Renal expression of NF-κβ and $CB_2$by western blot method was estimated by the method described in the Soetikno et al. Radioimmunoprecipitation assay (RIPA) buffer was used for protein extraction from kidney tissues.10% SDS-PAGE was prepared to separate protein samples in laemmli buffer, then transferred the proteins to a Poly-vinylidene fluoride (PVDF) membrane (0.45 mM, Merck Millipore, Germany). 5% skimmed milk was used to block the membrane for one hour. NF-κβ (NF-κBp65 Antibody Mouse monoclonal IgG1kappa (IgG1κ), Santa Cruz Biotechnology, Inc. USA) and β-Actin Antibody mouse monoclonal (IgG1κ) mouse Santa Cruz Biotechnology, Inc. USA) (loading control) was used as primary antibodies at dilution 1:1000 for 2 hours. Then was secondary antibody mouse (IgG1κ) binding protein conjugated to Horseradish Peroxidase (HRP) (Santa Cruz Biotechnology, Inc. USA) at dilution 1:1250 for 1 hour was treated with samples. Then used TBST to wash three times for 10 minutes. In a dark setting, X-ray film was used for enhancing chemiluminescence (ECL) imaging. The signal intensities of the expressed

proteins were assessed using Image Studio Lite version 5.2 software from LI-COR Biosciences in the United States (Soetikno et al., 2011).

Histopathological Examination:

Kidney tissue fixed in 10% v/v neutral buffered formalin and embedded in paraffin. Thin (5µm) transverse sections of kidney tissues were taken and stained with Mayer's hematoxylin and eosin stain (H.E. stain), Periodic acid–Schiff (PAS) stain and Trichrome stain [39]. The prepared slides were examined under a microscope to observe histopathological lesions if any.

**To study the effect of Combination of Beta-caryophyllene and L-arginine in an experimental model of diabetic Cardiomyopathy:**

Evaluation Parameters:

Body weight and heart weight

The body weight was measured at the end of the study. At the end of the study, animals were sacrificed and the heart was perfused with normal saline solution to remove blood. The heart was isolated and weighed to determine relative heart weight.

Biochemical Parameters:

Plasma glucose level was measured using commercially available kits. Serum samples were subjected to determine cardiac marker enzymes like creatinine kinase-MB (CK-MB), Lactate dehydrogenase – P (LDH-P), using the commercially available kit. The lipid profile was determined by measuring high-density lipoproteins (HDL), total cholesterol (TC), and triglycerides (TG) in serum samples as per the manufacturer's protocol. All the biochemistry parameters were estimated using a biochemistry analyzer.

TNF-α and IL-6 analysis by ELISA method:

Plasma samples were tested for TNF-α and IL-6 by the commercially available ELISA ( BT Bioassay).

Assessment of electrocardiogram (ECG) parameters:

After 4 weeks of treatment, rats were anaesthetized using terminal anaesthesia (Urethane, 1.2 g/kg, i.p.) for ECG parameters. The ECG was recorded using a Lead II configuration. The needle electrodes (29-gauge, 12 mm) were placed beneath the skin at the left inguinal (positive lead), right inguinal (neutral lead), and right axilla (negative lead). Electrodes were connected to the data acquisition system (AD Instrument, Australia) and responses were recorded for 5 minutes after stabilization of animals for 30 min. The heart rate (HR), R-R interval, QRS interval, QT interval, and corrected QT interval (QTc) were calculated from ECG recordings [40].

Assessment of hemodynamic parameters:

After recording of ECG parameters, hemodynamic parameters such as mean arterial pressure (MAP), systolic blood pressure (SBP), and diastolic blood pressure (DBP) were evaluated. The right carotid artery was cannulated with a polyethene catheter and connected with the physiological pressure transducer system. After stabilization of the animal, MAP, SBP and DBP were recorded with a data acquisition system (AD Instrument, Australia) [40,41].

Assessment of heart oxidative stress parameters:

Isolated heart tissues were homogenized in ice-cold phosphate buffer (0.1 M, pH – 7.4) by using probe homogenizer. The homogenate as such was used to determine a protein content [42], malondialdehyde (MDA) [43] and reduced glutathione (GSH) [44] and further processed to obtain post-nuclear and post mitochondrial supernatant. Catalase assay was performed using post-nuclear

supernatant obtained from homogenate [45]. The post-mitochondrial supernatant was used to perform superoxide dismutase (SOD) assay [46].

Western blotting:

The heart tissues were subjected to protein extraction with Radioimmunoprecipitation assay (RIPA) buffer. The protein estimation was done as described previously [42]. 40 µg of protein was separated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred on PVDF membrane (0.45 µm, Merck Millipore, Germany). Blocking was done with freshly prepared 5%w/v non-fat dried milk powder for 2 hours. Membranes were treated with primary antibodies (NF-κβ, CB2 and β-actin) at dilution 1:1000 for two hours. After subsequent washings membranes were treated with secondary antibody (m-IgGk BP-HRP) at dilution 1:1250 for one hour. The bands were visualized with Clarity Western ECL Substrate on X-ray films. The expressions were analyzed with Software Image Studio Lite version 5.2.

Histopathological Examination:

Heart tissue fixed in 10% v/v neutral buffered formalin (NBF) were further embedded in paraffin. Thin (5µm) transverse sections were taken with a rotary microtome (Leica, Germany). The sections were stained with Mayer's hematoxylin and eosin stain (H.E. stain) and Trichrome stain. The prepared slides were examined under the microscope to observe histopathological lesions if any and collagen deposition.

**To study the effect of Combination of Beta-caryophyllene and L-arginine in an experimental model of diabetic neuropathy:**

DCM was induced with single-dose administration of STZ (55 mg/kg, i.p.). Animals with plasma glucose levels (PGL) higher than 250 mg/dl were included in the study. After six weeks, animals were randomized into different groups containing eight animals each. One group of non-diabetic animals was kept as normal control while one group of diabetic animals was kept as diabetic control, Other groups received Insulin 2 IU, Pregabalin (30 mg/kg) BCP (200 mg/ kg), LA (100 mg/kg), COM1- BCP (100 mg/kg) and LA (100 mg/kg) and COM2- BCP (200 mg/kg) and LA (200 mg/kg) After 28 days of treatment following parameters were evaluated.

Biochemical Parameters:

The body weight was measured at the end of study. Blood was collected by retro-orbital puncture method. Whole blood was centrifuged at 2500 rpm for 15 minutes at 4°C to separate the plasma and stored at temperature -20°C till further study. Plasma glucose level was measured using commercially available kits.

Hot plate test:

Eddy's hot plate test was carried out to study the effect of treatment on the thermal nociceptive threshold in diabetic rats. After 28 days of treatment, animals were placed on a hot plate (IITC Life Sciences, USA) maintained at 55±0.5°C. The time required for the first response (Flickering, licking of hind paw or jumping) was recorded. The cut off time was set to 15 seconds to avoid tissue damage [17]

Tail immersion test:

The tail immersion test was carried out on the 28$^{th}$ day of treatment to determine the thermal nociceptive threshold. Animals were acclimatized to restrainer 30 minutes before the experiment. The tail of the animals was completely immersed in hot water maintained at 55±0.5°C. The time

required to remove the tail from hot water was considered as response time. The cut-off time for tail to hot water was set to 15 seconds to avoid tissue damage [47].

Mechanical hyperalgesia:

Mechanical hyperalgesia was determined using the Randall-Selitto test to determine the mechanical nociceptive threshold. At the end of the study, animals were restrained in cloth slings and subjected to constantly increasing pressure stimulus with a pressure applicator to the dorsal surface of the hind paw. The maximum pressure at which an animal showed a response (paw withdrawal, squeaking or struggling) was recorded with a digital meter. The cut-off pressure was set to 400 g. Readings were recorded in triplicate separated by at least 20 minutes intervals for each rat and mean values were considered as final readings [48].

Mechanical allodynia:

Mechanical allodynia was recorded using the Von Frey test to determine the nociceptive threshold to innocuous stimuli. At the end of the study, animals were transferred to the elevated cage with a wire mesh bottom. Animals were allowed to acclimatize with the cage for 20 minutes. A constantly increasing pressure stimulus was applied with an electronic Von Frey pressure applicator attached with a rigid tip to the ventral surface of the hind paw. The maximum pressure at which the animal showed a response (paw withdrawal or brisk lifting) was recorded with digital. The cut-off pressure was set to 400 g. Readings were recorded in triplicate separated by at least 20 minutes intervals for each rat and mean values were considered as final readings [49].

Oxidative stress parameters

At the end of the study, sciatic nerves were carefully isolated and homogenized in ice-cold phosphate buffer (0.1M, pH – 7.4) by using a probe homogenizer. Homogenate was further divided

into different aliquots to get a post mitochondrial fraction and a post-nuclear fraction [39]. The protein concentration was determined in tissue homogenate with the method described earlier [42]. The lipid peroxidation was determined by assessing the malondialdehyde (MDA) level [43]. The tissue sulfhydryl groups were determined by accessing reduced glutathione (GSH) levels [44]. Catalase activity was determined by accessing moles of hydrogen peroxide decomposed per minute [45]. The superoxide dismutase (SOD) activity was determined as per the method described earlier [50].

Western blotting:

Sciatic nerve tissues were minced separately with RIPA buffer to extract proteins and protein content was determined as described previously [42]. 40 μg of protein were loaded and separated on 10% acrylamide gel with electrophoretic transfer cell. Protein transfer was carried out on Polyvinylidene difluoride (PVDF) membrane using a tris-glycine transfer buffer containing 20% methanol at constant volt (100 V) for one hour. Blocking was carried out with freshly prepared 5% non-fat dried milk powder in Tris buffer saline (TBS) solution for 2 hours. Primary antibody treatment of NF-κβ and $CB_2$ and β-actin was given at dilution 1:1000 for two hours. Three washings of 15 minutes each were performed with TBS containing 0.1% tween 20. Secondary antibody treatment (m-IgGk BP-HRP) was given at dilution 1:1250 for one hour. After washings, the bands were visualized with enhanced chemiluminescence technique on X-ray films. The relative expressions were analyzed by measuring optical density with Software.

Histopathology Study:

The sciatic nerves were carefully isolated and fixed in neutral formalin buffer and embedded in paraffin wax. Thin sections of 5 μm were taken with microtome and sections were stained with Mayer's hematoxylin and eosin (HE) stain. The slides were observed for pathological changes.

Statistical Analysis:

Values were expressed as mean. GraphPad Prism ver. 5.00 for Windows was used to analyze the data. Analysis of variance (ANOVA) followed by Dunnett's test was used to determine the statistical significance of the difference between the groups. The values were considered to be significantly different when the *P*-value was less than 0.05.

**Results:**

**To study the effects of Combination of Beta-caryophyllene and L-arginine on inflammatory markers on RAW 264.7 cell lines:**

LPS treatment showed cytotoxicity to RAW 264.7 cell lines after 24 hrs. Whereas, alone treatment as well as concomitant of BCP and LA did not exhibit toxicity as compared to control cells at 100, 200 and 500 μM concentrations. The above doses of drugs were used evaluate the anti-inflammatory activity in cells. Both levels of IL-6 and TNF- α were discovered significantly higher after LPS treatment against the control group. To the contrary, BCP with LA treatment showed significantly declined in the levels of IL- 6 and TNF-α($p<0.001$) higher concentrations of BCP and LA combined resulted in a greater decrease in IL-6 and TNF-α levels, respectively.

**To study the effect of Combination of Beta-caryophyllene and L-arginine in an experimental model of diabetic nephropathy:**

The treatment with COM1 and COM2 significantly increased body weight and decreased the relative kidney weight when compared with disease control animals. Plasma glucose and creatinine and BUN levels are significantly decreased in the COM1 and COM2 treatment. The treatment with COM1 and COM2 also reduced proteinuria, albuminuria and improved the GFR in the diabetic animals. Anti-oxidant enzymes were significantly improved and lipid peroxidation was

significantly reduced in the COM2 group when compared with the disease control group. Inflammatory markers such as TNF-α and IL- 6 levels were significantly reduced in the COM1 and COM2 treated animals. Nf-κβ expression in the kidney was reduced and CB2 expression was improved in the COM2 treated animals when compared with the disease control group. Histopathological study showed combinational treatment protects the kidney and prevented the progression of kidney damage.

**To study the effect of Combination of Beta-caryophyllene and L-arginine in an experimental model of diabetic Cardiomyopathy:**

After 4 weeks of treatment of COM1 and COM2 showed a significant increase in the body weight and a significant decrease in the plasma glucose levels as well as relative heart weight when compared with the disease control group. Cardiac markers such as CK-MB and LDH levels were significantly decreased in the COM1 and COM2 treated animals when compared to the disease control group, lipid profiles also normalize in the COM1 and COM2 groups. Furthermore, COM1 and COM2 markedly normalize the SBP, DBP and MAP when compared to the disease control rats. The inflammatory markers such as TNF-α and IL-6 were significantly reduced in the COM2 group. After 28 days of treatment, COM1 and COM2 showed a significant increase in GSH, CAT and SOD enzymes when compared with the disease control group. MDA levels were significantly decreased in the COM1 and COM2 treated animals when compared with the STZ treated group. The western blotting results showed that COM1 and COM2 treatment significantly inhibited the Nf-κβ expression and increased the CB2 receptor expression.

**To study the effect of Combination of Beta-caryophyllene and L-arginine in an experimental model of diabetic neuropathy:**

The treatment of COM1 and COM2 showed a significant reduction in the plasma glucose levels and a significant increment in the bodyweight when compared with the disease control group. COM1 and COM2 treatment significantly reduced the thermal hyperalgesia, mechanical hyperalgesia and mechanical allodynia in diabetic rats. COM2 treatment significantly reduced oxidative stress and lipid peroxidation by increasing GSH, catalase and SOD levels and decreasing MDA levels in sciatic nerves. The NF-κβ expression also decreased in the sciatic nerve of COM 1 and COM2 treated animals when compared with the disease control group. Sciatic nerve degeneration was reduced in the COM1 and COM2 treated animals.

**Conclusion:**

We found that a combination of BCP (200 mg/kg) with LA (200 mg/kg) resulted in the lowering of plasma glucose levels, cardiac enzyme markers, Kidney markers and normalizing systolic and diastolic functions as well as decreasing in the inflammatory signalling cascades in diabetic cardiomyopathy, Neuropathy and nephropathy when compared with the individual therapies. . In view of these results, the combination of BCP (200 mg/kg) with LA (200 mg/kg) could be potentially used as a promising therapeutic drug combination to ameliorate diabetic complications.

**Publication Details:**

| Sr. No. | Details of publications | Impact factor | Status |
|---|---|---|---|
| **1** | Vivek S. Kumawat, Ginpreet Kaur, (2019). Therapeutic potential of Cannabinoid receptor 2 in the treatment of Diabetic Mellitus and its complications, *European Journal of Pharmacology,* 862;11; 1-5 | 3.170 | Published |
| **2** | Vivek S. Kumawat, Ginpreet Kaur (2021), CB2 receptor agonist and L- arginine combination attenuates diabetic cardiomyopathy in rats via Nf-κβ inhibition, *The Canadian Journal of Physiology and Pharmacology.* | 1.98 | Published |
| **3** | Insulinotropic and antidiabetic effects of β-caryophyllene with l-arginine in type 2 diabetic rats (2020), *Journal of food and biochemistry, 44; 2; 1-5* | 1.64 | Published |
| **4** | Vivek S. Kumawat, Ginpreet Kaur, Combination of Beta-caryophyllene with L-arginine ameliorates type 2 diabetes in rats through upregulation SIRT1 expression. *Biotechnic & histochemistry* | 1.75 | Under Review |
| **5** | Vivek S. Kumawat, Ginpreet Kaur, Cannabinoid receptor 2 (CB2) agonists and L-Arginine ameliorate diabetic nephropathy | 3.6 | Accepted |

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|---|---|---|---|
| | in rats by suppressing inflammation and fibrosis through NF-κβ pathway. Naunyn-Schmiedeberg's Archives of Pharmacology | | |

**Book chapter**:

Kumawat VS, Kaur G., Therapeutic Interventions of Endocannabinoid Signaling in Obesity-Related Cardiovascular Dysfunction (2021), Biochemistry of Cardiovascular Dysfunction in Obesity, Vol-20, Page No-267-281.

**Presentations:**

1) Kumawat VS, Kaur G; Innovative approach to target type 2 diabetes mellitus: Cannabinoid receptor 2 agonists combined with l-arginine, 5th IUPHAR World Conference on The Pharmacology of Natural Products at NIN Hyderabad, India, 04th – 07th Dec 2019.

2) Kumawat VS, Kaur G; Evaluation of the anti-diabetic activity of the combination of Beta-Caryophyllene and L-Arginine in streptozotocin-induced diabetic rats, Annual Conference of Indian Pharmacological Society (IPSCON - 2017) at SPP School of Pharmacy and Technology Management, NMIMS, SVKM'S Mumbai, India 15th -17th Feb 2018.

3) Kumawat VS, Kaur G. New approach towards Treatment of Diabetes Mellitus with Beta-Caryophyllene and L- arginine Potential Combination at Annual Conference of Indian Pharmacological Society (IPSCON - 2017) at SPP School of Pharmacy and Technology Management, NMIMS, SVKM'S Mumbai. 15th – 17th February 2018.

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Vivek S. Kumawat — Candidate

Dr. Ginpreet Kaur — Supervisor